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GENE TRANSFER SYSTEMS

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Summary. *The ability to introduce isolated DNA into eukaryotic cells has tremendous influence on advance in molecular biology. During the past decades, a wide repertoire of gene transfer techniques has evolved. Recently, with the development of attractive strategies for gene therapy, successful gene delivery has gained importance again and become a major challenge in this field. The purpose of this article is to summarise available gene transfer systems, their principles, advantages and limitations.*

Key words: *Gene transfer, viral vectors, non viral vectors*

Introduction

Over the past decades considerable progress has been made in the development of a variety of techniques and reagents for the delivery of macromolecules into eukaryotic cells.

Nowdays, it is possible to transfer DNA, RNA, oligonucleotides, proteins and small molecules into almost all types of cells. This review is focused on gene transfer systems. Started as a research tool for molecular mechanisms of gene expression and regulation, the gene transfer technology has recently found its chalenging application in gene therapy of inherated disease, cancer and viral infections (like AIDS) (1-3). Gene therapy implies any clinical therapeutic procedure in which recombinant gene(s) is intentionally introduced into human somatic cells to replace protein with a genetic defect or to interfere with patological process of an ilness (4). When a gene, i.e. DNA molecule encoding a protein, is transferred to the nucleus of the target cell, it will be used as a template for mRNA synthesis which in turn will lead to the production of therapeutic protein which is absent or mutated in a patient's cell. This simple principle of gene transfer can be applied in practice by combining (or inserting) a gene with (in) carrier molecules (or vectors) that allows the DNA to be transported safely and efficiently into the nuclei of the target cells. The quest for an ideal carrier/vector for gene transfer is still ongoing.

Generally, there two main approaches: the one utilizing biological, viral vectors and the other utilizing either chemical or physical methods to introduce gene of interest into target cells. The first one, the viral gene transfer is a viral-mediated process reffered to as an infection. The second, non-viral gene transfer involves treatment of cell by chemical or physical means and the process itself is named transfection. Gene delivery by infection is more complicated than transfection. Infection requires more steps and more times than does transfection and biosafety issues may also arise, depending on the virus used. A much safer alternative to infection - transfection, is also faster and requires only a few reagents including plasmid DNA containing the gene of interest under the control of a strong cell-specific promotor. However, inefficient gene delivery and poor sustained gene expression are its major drawbacks.

Transfection can be categorized into two major types: transient and stable. Transient transfection is temporary, i.e. expression of foreign gene lasts for several days and is lost as DNA never integrates into the host cell DNA. In contrast, stable transfection occurs with a lower frequency (10 to 100 – fold lower), but expression is maintained for the long term because the foreign DNA does integrate into the host genome.

This paper covers all available viral and non-viral gene transfer agents.

Viral gene transfer systems

The most effective gene transfer system available today exploits an over million of years evolved gene transfer capability of animal viruses. A growing understanding of virus life cycles has enabled development of recombinant viruses with some or all of the genes replaced by the relevant therapeutic gene (5). Resulting recombinant viral vectors are either short-term (lytic viruses, such as baculovirus, adenovirus or alphaviruses) or long-term (retroviruses, lentiviruses, adeno-associated viruses, Epstein-Barr virus) in their expression of transffered therapeutic gene material (6). For the sake of conciseness, we will briefly discuss characteristics of retroviral and adenoviral vectors only.

The most frequently used system is the one based on retroviruses. 34,1 % out of 636 approved ongoing gene therapy clinical trials with 50,2 % out of 3494 patients

involved utilize recombinant retroviruses (7). Retroviruses are RNA viruses which use viral enzymes and cellular transcription machinery to copy their own genome and integrate it into the host chromosome of actively dividing cells. Thanks to integration, this system achieves long-term expression of therapeutic gene (8).

The backbone of Moloney Murine Leukemia Virus (Mo-MuLV) has been favored for years for the design of retroviral vectors. Since its first application, this system is extensively engineered to improve the efficiency and safety of gene transfer (9, 10).

There are only few drawbacks of retroviral system which should be solved in the future. These include retrovirus inability to infect non-dividing cells (e.g., muscle, brain, lung, liver, quiscent stem cells), random integration of its genom with associated risk of insertional mutagenesis, problems with its production at high titres, its limited capacity for therapeutic gene (maximum size og gene insert is 8 kb) and the possibility of generation of new recombinant replication competent virus, i.e. wild type retrovirus (6, 11, 12).

Recently, another subgroup of retroviruses, lentiviruses, has been considered as a promissing tool in gene transfer. They share all the standard properties of retroviruses, but in addition they have the capacity to tranduce non-dividing cells (6, 13, 14).

On the other hand, linear double-stranded DNA viruses, adenoviruses are able to infect both dividing and non-dividing cells (6, 15). This makes them attractive for gene transfer applications, together with a fact that they can be produced as high viral titres. However, they do not integrate into the host cell genome and thus gene expression following adenoviral gene transfer is short lived. Also, there is a probleme with potential immune response of the patient which leads to the elimination of therapeutic antigenic cells (16).

Non-viral gene transfer systems

Non-viral gene transfer agents offer several potential advantages over recombinant viruses. They are non-infectious, relatively non-immunogenic, have low acute toxicity, can accommodate a large DNA plasmid and may be produced simply on a large scale. They are limited by their lower gene transfer efficiency than viruses and transient gene expression. Numerous non-viral gene transfer systems have been proposed including naked DNA, various chemical agents and physical methods (17).

A naked DNA injection into local tissues (smooth muscle, tumour, etc.) or systemic circulation is the simplest approach, but the last one has to deal with the problem of rapid degradation of DNA in the bloodstream. Otherwise, naked DNA is stable *in vivo* and long-term expression of the encoded protein is seen without chromosomal integration. This technique has been applied to DNA vaccination or cytokine gene therapy for various diseases, including infections, autoimmune disorders and cancer (18).

Gene transfer by chemical methods

Historically, the first succesfull gene transfer was achieved by utilization of a chemical reagent, DEAE dextran (19). Ever since, different reagents are involved in creating a chemical environment that facilitate DNA uptake by cells.

The positively charged polymers, as DEAE – dextran, polybrene, polyethylenimine and dendrimer, complex with negatively charged DNA molecules forming so called polyplex (20, 21). An enhanced ionic attraction between the net positive charge on the polycation-DNA complex and the negative charge on the cell surface enable the DNA binding and entrance into the cell by endocytosis by as yet uncharacterized pathways and/or inhibit the action of nucleases (20). Several parametars, such as: the number of cells, polymer concentration, transfected DNA concentration and transfection duration should be optimized for a given cell line. Complexed-DNA delivery with DEAE-dextran could be improved by osmotic shock using DMSO or glycerol or tretment with chloroquine (20). DEAE-dextran, in distinction from other cationic polymers, is limited to use in transient transfections only (20).

The most widely used technique is calcium phosphate co-precipitation with DNA. The mixing of calcium chloride, DNA and phosphate buffer precipitates extremely small, insolubile particles of calcium phosphate containing condensed DNA (22). Althought the mechanism of this type of transfection has not been characterized in detail, it is presumed that the calcium phosphate-DNA complexes adhere to the cell membranes leading to phagocytosis (23).

Calcium phosphate-mediated transfection can be applied for transient and stable transfection experiments very successfully to most adherent cells and some cells in suspension. Naturaly, for efficient transfection of the cells in question, optimal factors have to be determined (amount of DNA in the precipitate, the length of time for: precipitation reaction and exposure of cells to the precipitate) (24).

Promissing new transfection method involves cationic lipids or liposomes. A cationic headgroups interact strongly with negatively charged phosphates on DNA, forming DNA-cationic lipids complexes, termed lipoplexes (25). It is believed that two to four liposomes associate with a single plasmid DNA of about 5 kb (26). Net positively charged lipoplexes bind to negatively charged sialic acid residues on the cell surfaces and thus promote the passage of DNA through cell membrane. Evidence exists that the mechanisms of DNA delivery is throught endosomes and lysosomes by microtubule mediated pathway (27, 28).

Presently, numerous liposome reagents (LipofectAMINE™ 2000, LipofectAMINE PLUS™, LipofectAMINE™, DMRIE-C, CellFECTIN®, LipoFECTIN® Reagent - Invitrogen; Effectene Transfection Reagent - Qiagen; TransFastTM, TfxTM – 10, 20 or 50, Transfectam - Promega) are commercially available and highly

effective for transient or stable, both in vitro and in vivo, gene transfer to a broad range of cell types. Lipofection is simple to perform even with cell lines normally resistant to transfection by other methods and ensures consistently reproducible results. Also, it is more efficient then traditional calcium phosphate and DEAE-dextran transfection (5 and greater than 100 times, respectively) and requires smaller amounts of DNA (26). But one should keep in mind that three primary parameters - the concentration of lipid and DNA and incubation time of cells with the liposome - DNA complex, should be systematically examined to obtain optimal transfection frequences with particular cell line (24, 29).

The latest generation of liposome is designed with surface-associated targeting information (e.g., monoclonal antibodies, glycolipids, alkylphospholipids, proteins or vitamins) and succesfully applied to facilitate targeted gene delivery (30, 31, 32, 33).

Gene transfer by physical methods

Physical methods for this purpose have been developed more recently. They have become increasingly popular, although their application demands special equipment.

The phenomenon of introducing DNA into cells by application of short electric field pulse is termed electroporation or electropermeabilization. In essence, electroporation makes use of the fact that the cell membrane acts as an electrical capacitor which is unable (except throught ion channels) to pass current. The applied high-voltage electric field results in transient elevation of the transmembrane voltage to approximately 1 V. Consequently, a dramatic membrane reorganisation takes place – it breaks down and creates aqueous pathways or electropores. The DNA presumably diffuses into the cell through these pores which are believed to subsequently shrink and disapear. Although most electropores close rapidly, some may remain open for hours (34).

Electroporation provides a valuable alternative to chemical and other physical methods that may be ineffective or toxic when transforming certain cell types. It has been reported that electroporation is more efficient than traditional calcium phosphate transfection by as much as three orders of magnitudes (35). The efficiency of electric field-mediated gene transfer is influenced by different physical (the strength of the applied electric field, the length of the electric pulse, temperature) and biological (number of cells, conformation and concentration of DNA, ionic composition of the medium) factors (36, 37). The exact conditions for optimal electroporation must be determined for each cell type, since the pulse must penetrate cells that differ in diameter and membrane/cell wall composition. The objective is to define physical and biological parameters under which the cell viability is about 20-50% (36).

Besides *in vitro* transient or stable transfections, electroporation is lately frequently applied in *in vivo* electro-gene therapy of electrode-accessible tissues and malignances in animal models (38, 39)

The latest transfection technology also includes mechanically based methods such as microinjection, bead transfection and biolistic particle delivery. The most direct method – microinjection introduces DNA directly to the nucleus using a fine needle (40).

Bead transfection combines the principle of physically producing breaks in the cellular membrane with the use of beads. It involves brief incubation of adherent cells with glass beads in a solution containing the DNA to be transferred. The efficiency of this rapid technique depends on: the concentration of DNA in a solution, the timing of the addition of DNA, the size and condition of the beads and the buffers utilised (41). A recently developed new type of beads, Immunofect beads, can be targeted to make holes in a specific type of cells and thus, this particular bead transfection process is renamed as immunoporation (42).

Biolistic particle delivery, gene gun or DNA-coated particle bombardment utilizes heavy metal microparticles (tungsten or gold, 1-5 μm in diameter) accelerated to the sufficient velocity to penetrate the target cells. Within the cell, particles are visible (under a microscope) and they gradually release DNA. This technology is suitable for *in situ*, *in vivo*, *ex vivo* and *in vitro* transient and stable gene delivery (43, 44).

Different commercially available devices (Helios Gene Gun System, Biolistic PDS-1000/He System – BIO-RAD) provide the motive force (helium pulse) for launching and delivering of DNA-coated particles into virtually any target (organ, tissue or single cell). Fine tuning of motive force by changing the helium pressure range results in changed velocity and final distribution of microparticles in target. Also, varying the particle density and size can affect bombardment efficiency (45).

Conclusions

Many studies and advances have been achieved in the field of gene transfer, but the main obstacle, poor efficiency, still remains.

Despite widespread use of the mentioned chemical and physical gene transfer methods and heaps of literature filled with successful experiments, one still faces a problem of optimization for the cell type being studied. The optimal conditions for each particular transfection method, described before, should be determined experimentally.

In the case of gene expression analysis, besides traditional calcium phosphate coprecipitation method, lipofection and electroporation are the most popular because of their simplicity and rapidity. Although more effective in stable transfer, tedious microinjection and viral injection are rarely used in this type of study.

The high expectation of gene therapy requires efficient selective delivery and sustained expression of a therapeutic gene into the tissues of a human body. The "magic" vector should be targeted, protected from degradation and immune attack and safe for the recipient and the environment. Moreover, it should express the

therapeutic gene for as long as required, in an appropriately regulated fashion (46).

Viral vectors are still the most prominent vehicles for gene therapy. There have been three major chalenges in this field. One is to generate vectors capable of carrying sizable regions of genomic DNA. Another is to increase the safety and decrease the immunogenicity of such vectors. The last is to restrict infection of vectors to a predetermined target cell (47).

For the time being, few non-viral methods (liposomes, electroporation, gene-gun) are promising for gene therapy/gene marking protocols and are providing research tools to improve the gene transfer efficiency and gene expression. The use of non-viral rather than viral methods for gene delivery has several advantages, including nonimmunicity and potential for transfering and expressing large pieces of DNA into cells. The success of non-viral gene transfer will be greatly dependent on the ability to design systems with reduced toxicity.

Furthermore, new strategies which combine the best properties of non-viral and viral techniques are reported (48-52). This is an area of growing opporunities.

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SISTEMI ZA TRANSFER GENA

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Odsek Biologija sa ekologijom Prirodno-matematičkog fakulteta, Niš

Kratak sadržaj: *Mogućnost unošenja DNK u eukariotske ćelije je imala ogroman uticaj na napredak molekularne biologije. Tokom proteklih decenija, razvijen je široki repertoar tehnika sa ovom svrhom. U skorije vreme, sa pojavom privlačnih strategija za gensku terapiju, problemi uspešnog transfera gena postaju ponovo aktuelni. U ovom radu je dat pregled metoda za transfer gena, njihovih prednosti i ograničenja, kao i mehanizama na kojima su zasnovane.*

Ključne reči: *Transfer gena, virusni vektori, nevirusni vektori*